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THE STRUCTURE OF THE LOW MOLECULAR-WEIGHT GLUCANS ISOLATED FROM THE SIPHONOUS GREEN ALGA *Caulerpa simpliciuscula*

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ABSTRACT

A β-D-glucan of low molecular weight isolated from the marine alga *Caulerpa simpliciuscula* has been shown to contain 30 glucose residues. At least 27 of these are β-D-(1→3) linked. There are 1–2 β-(1→6) branches per molecule, with a maximum of 4 D-glucose residues per side chain. As normally isolated, this glucan is associated with a soluble (1→4)-α-D-glucan (soluble starch) of the same molecular weight, in the ratio of 3 molecules of β-D-glucan per molecule of α-D-linked glucan.

INTRODUCTION

The siphonous green alga *Caulerpa simpliciuscula* produces soluble glucans in significant amounts during photosynthesis[1,2]. Together with amylopectin-like compounds[2], these appear to be the important storage carbohydrates in the plant. The soluble glucans are mainly β-D-(1→3)-linked[3]. A soluble amylopectin[4] and a series of sulphated polysaccharides[5] have been found in *Caulerpa sediodes*, but soluble (1→3)-β-D-glucans are not commonly found in members of the Chlorophyceae, although present as storage carbohydrates in the Phaeophyceae[6,7] and Bacillariophyceae[8–10]. More recently, a soluble (1→3)-β-D-glucan has been found in a green alga of another genus, *Codiolum pusillum*[11]. Soluble (1→3)-β-D-glucans have also been found in a variety of species of fungi, particularly in the genus *Phytophthora*[12,13]. It therefore appeared possible that one or more of the glucans in this class may have some particular function associated with the a-cellular anatomy, such as participation in the wound-healing process. In this paper we give details of the structure of the low-molecular-weight glucan component.

EXPERIMENTAL

General methods. — Solvents were removed either by rotary evaporation below 40° or by freeze drying. Paper chromatography was conducted as described previously[1,3] with the following solvent systems: (*a*) 7:7:6 (v/v) isoamyl alcohol–pyridine–water; (*b*) 3:1:3 (v/v) ethyl acetate–acetic acid–water (upper phase). The mass of carbohydrate was measured by using a modified phenol–sulphuric acid

method[14] with D-glucose or laminaran as standards. Gas–liquid chromatography was performed by using stainless-steel columns, 6 ft. × 1/8 in., with nitrogen as the carrier at a flow rate of 40 mL per minute. Four types of column were used; (*a*) 3% ECNSS-M on Anakrom Q, 100–200 mesh; (*b*) 3% OV-225 on Chromosorb W (HP), 80–100 mesh; (*c*) 20% BDS on Gas Chrom W, 70–80 mesh; and (*d*) 3% OV-1 on Gas Chrom W (HP), 80–100 mesh.

I.r. spectra were recorded with the glucan in potassium bromide discs, for samples that had been dried *in vacuo* over phosphorus pentaoxide for one week prior to the measurement. Optical rotations were measured with 1% solutions on a Perkin–Elmer 141 polarimeter.

Purified glucan (25 mg), after prolonged drying *in vacuo* over phosphorus pentaoxide, was analysed for C, H, N, O, S, by the C.S.I.R.O. Microanalytical Service.

Preparation of glucan fraction. — Freshly collected *Caulerpa simpliciuscula* tissue was killed in boiling ethanol, homogenised, and extracted 3 times in cold water, the homogenate being filtered through a sintered-glass filter. The water extracts were combined with the original ethanol used, so that the combined liquor contained 25% ethanol by volume. The solution was kept for 3 days at 4° and then filtered through diatomaceous earth and concentrated to 2% of its initial volume. This extract was then dialysed against running water overnight at 4°. The dialysate was diluted with one-half its volume of 95% ethanol and any precipitate removed by centrifugation. The ethanol concentration of the supernatant was then adjusted to 90%, and the precipitate containing the crude glucan collected.

This fraction was de-ionised by sequential passage through the ion-exchange resins Dowex-50 (H^+ form) and Dowex-1 (HCO_2^- form). The neutral material was then fractionated by using an Amicon PM-10 membrane (retention limit 10,000 daltons) and the filtrate passed through a column of poly(acrylamide) gel (Biogel P-30). The material migrated as the single peak; it was collected, freeze dried, and used as the source of the low-molecular-weight glucan described in this paper. The average yield was 30 mg/kg of alga (fresh, drained weight).

In developing this method, radioactive glucan labelled during photosynthesis in $^{14}CO_2$, isolated and purified as described in previous work[1,3], was added as a marker. In the final chromatographic step, constant specific activity of the glucan fraction was obtained and the recovery of added radioactivity was 85% or higher.

Sugar analysis. — The glucan (2 mg) was hydrolysed in 0.25M sulphuric acid for 16 h at 100° in a sealed tube. The acid was removed by using Dowex-1 resin (CO_3^{2-} form), and the hydrolysate trimethylsilylated by the method of Sweeley *et al.*[15]. The products were separated on the OV-1 column, programmed from 135–250° at 3.3° per min.

Complete enzymic hydrolysis was also effected as described previously[3] and products were separated by paper chromatography with solvent system *a*, and their mass[3] and radioactivity measured[1]. In the final purification step, the sample was

incubated with alpha amylase from *Bacillus subtilis* (Calbiochem, Lot No. 201496) as described under the section Enzymic Hydrolysis.

Molecular weight determination. — *A.* Ultracentrifugation was performed with a Beckman Spinco Model E analytical ultracentrifuge. Velocity sedimentation was conducted at 59,000 r.p.m. with a solution of glucan (7 mg/mL) in 0.1M acetate buffer at pH 5.3.

B. Gel filtration was effected on a Bio-gel P-30 column (100 × 1 cm) with Dextran T-10 (mol. wt. 9,300), T-20 (mol. wt. 22,300), and laminaran from *Laminaria hypoborea* (mol. wt. 3,900) as standards. Water containing 0.02% sodium azide was used as the eluant.

Methylation analysis. — The glucan was pre-methylated by the Haworth procedure[16], dried (phosphorus pentaoxide) and then re-methylated by two successive Hakomori methylations without isolation of the intermediate products[17]. The methylated glucan was separated by dialysis[18] and dried over phosphorus pentaoxide *in vacuo*; i.r. spectra were used to determine the presence of free hydroxyl groups. Fully methylated glucan was hydrolysed sequentially in 90% formic acid, and then 0.25M sulphuric acid[19]. The methylated derivatives were converted into alditol acetates[20,21] and resolved isothermally by g.l.c. at 170° with columns (*a*) and (*b*). The relative proportions of mass in each component were calculated by the effective carbon-ratio method[22].

Methyl glycosides were also prepared from the neutralised, aqueous filtrate obtained by hydrolysis of the methylated glucan. Freeze-dried sample (5 mg) was dried over phosphorus pentaoxide dissolved in 2 mL of 4% hydrogen chloride in methanol, and the solution heated for 16 h at 100° in a sealed tube. The acid was removed in a desiccator over dry sodium hydroxide, and glycosides were resolved by g.l.c. at 160° with column (*c*).

Periodate oxidation. — One series of oxidations was performed for 96 h in the dark at 4° in a 0.02% solution of sodium metaperiodate containing 0.15% of glucan. At intervals, aliquots were removed and the periodate consumption measured by determining[23] the change in absorption at 223 nm. In addition, production of formic acid was measured by titration of aliquots against mM sodium hydroxide (CO_3^{2-}-free). A second series of oxidations was performed in the presence of 10mM acetate buffer, pH 5.2, during 128 h. The reaction was then stopped with ethylene glycol and the oxidised polymer reduced with an excess of sodium borohydride. The products were separated on a column of Biogel P-4 (superfine) pre-equilibrated with water. The void-volume peak was collected, freeze-dried, and a portion subjected to a second periodate oxidation, while the remainder was subjected to mild hydrolysis with 0.3M sulphuric acid for 16 h at 22°. The products were then separated by paper chromatography using systems (*a*) and (*b*) and their mass and radioactivity determined.

Alkaline degradation. — This was performed with 8 mg of glucan, as described by Young *et al.*[24]. At various times, samples were removed, neutralised with hydrochloric

acid, and the total carbohydrate remaining was measured by the phenol–sulphuric acid method[14].

Enzymic hydrolysis. — The following enzymes were used in structural characterization of the glucan. (*a*) Alpha amylase was obtained from *Bacillus subtilis* (1260 AU/mg protein). Incubations (5 mg of glucan in 300 μL of 10mM maleate buffer, pH 6.9, 630 units enzyme) were performed during 24 h at 37°. Samples were removed at intervals and the products separated by paper chromatography (system *a*). After 24 h, the remainder of the mixture was chromatographed on a column (100 × 1 cm) of Biogel P-30 and the radioactive and the carbohydrate-positive peaks were located.

(*b*) (1→3)-β-D-Glucanase was prepared from *Nicotiana glutinosa*[25,26]. Incubations (2 mg of glucan in 200 μL of acetate buffer 10mM, pH 5.0, 50 μL of enzyme) were continued for 48 h at 30° and samples removed at intervals and separated as described for the *B. subtilis* enzyme. At the completion of the incubation, the remaining mixture was separated on a column (100 × 1 cm) of Biogel P-4 (superfine) and radioactivity and carbohydrate content of the eluate were measured.

(*c*) (1→3)-β-D-Glucanase was prepared from *Euglena gracilis* var. *bacillaris*[27]. Incubations (6 mg of glucan, in 600 μL of 10mM acetate buffer, pH 5.2, with 350 units of enzyme activity) were continued for 44 h at 35°. Samples were removed at intervals and analysed for the presence of reducing groups[28]. At the completion of the incubation, the mixture was separated on Biogel P-4 as already described. The fractions containing discrete peaks were then rechromatographed on paper (solvent system *b*).

RESULTS

The purified glucan sedimented as a single, broad peak with a sedimentation coefficient of 1.15 during velocity-sedimentation in the ultracentrifuge. A molecular weight of 5030 ±400 daltons was determined by using the Archibald method[29], (31,410 r.p.m.) and taking 0.67 ±0.1 $mg.g^{-1}$ as the partial specific volume. This value was independent of the concentration (3–8 $mg.mL^{-1}$) of glucan and of the ionic strength (0–0.1M) of the solvent. A value of 5000 ±300 daltons was calculated from gel chromatography, where the glucan was also eluted as a single, symmetrical peak in which mass and radioactivity were coincident. These molecular weights indicate a d.p. of 30 ±3.

Elemental analysis of the purified glucan indicated $C_1H_{1.8}O_{0.9}$. Its i.r. spectrum showed the following bands attributable by comparison with published spectra[30,31]. A shoulder at 890 cm^{-1} is characteristic of all β-linked glucans. Bands at 1370, 1200, 1155, 1075, and 1040 cm^{-1} are characteristic of (1→3)-β-D-glucans and have been found in laminaran and paramylon[30]. However, there is a distinct band at 850 cm^{-1}, and a shoulder at 925 cm^{-1}; both of which are characteristic of (1→4)-α-D-linked glucans[31]. After treatment with alpha amylase, the shoulder at 925 cm^{-1} disappeared. The broad absorption at 1630 cm^{-1} is due to bound water[32] which persisted, even

TABLE I

G.L.C. ANALYSIS OF METHYLATED GLUCITOL ACETATES FROM *Caulerpa simpliciuscula* GLUCAN

Derivative: position of O-methyl group	*Deduced structural unit*	R_T		*Percent composition*
		Observed	*Expected*[32]	
2,3,4,6-Tetra-	Non-reducing end group	1.00	1.00	6.4
2,4,6-Tri-	(1→3) Linkage	1.93	1.95	70
2,3,6-Tri-[a]	(1→4) Linkage	2.45	2.50	19
2,4-Di-	(1→3→6) Branch	4.80	5.10	2.9
2,3-Di-	(1→4→6) Branch	5.10	5.39	1.7

[a]The 2,3,4-trimethyl ether from a (1→6) linkage co-chromatographs (R_T = 2.49) with the 2,3,6-trimethyl ether on this column.

TABLE II

G.L.C. ANALYSIS OF METHYLATED GLUCITOL ACETATES FROM *Caulerpa simpliciuscula* GLUCAN PRETREATED WITH ALPHA AMYLASE

Derivative: position of O-methyl group	*Deduced structural unit*	R_T		*Percent composition*
		Observed	*Expected*[32]	
2,3,4,6-Tetra-	Non-reducing end group	1.00	1.00	7.4
2,4,6-Tri-	(1→3) Linkage	1.92	1.95	87
2,3,6-Tri-[a]	(1→4) Linkage	2.33	2.50	1.2
2,4-Di-	(1→3→6) Branch	4.42	5.10	4.2
2,3-Di-[b]	(1→4→6) Branch		5.39	

[a]The 2,3,4-trimethyl ether from a 1→6 linkage co-chromatographs (R_T = 2.49) with the 2,3,6-trimethyl ether on this column. [b]Insufficient of this component was present to be observed above baseline noise. Another component (1.2%, a 2,6-dimethyl derivative) was found in this sample. As it was not found in other samples, it is concluded that it arose through under-methylation.

after the most extensive drying. This result was confirmed by exposing a dried sample to D_2O, redrying and observing the band-shift from 1630 to 1570 cm^{-1}.

Methylation analysis. — The methylated glucitol acetates present (Table I) confirmed that the major linkage-type was (1→3), and the presence of the 2,4-dimethyl ether provided direct evidence for the existence of a (1→3→6)-branch, as proposed from previous results[3]. It was not possible to resolve the 2,3,6- and 2,3,4-tri-methyl ethers on the columns used, but separate analysis of the methyl glycosides showed that there were no (1→6) linkages other than at the branch point. Therefore, the methylated glucitol acetate having R_T 2.45 can only be due to the presence of 2,3,6-trimethyl ethers originating from (1→4)-linkages present in the sample. The proportion (6.4%) of 2,3,4,6-tetra-*O*-methylglucitol acetates observed, agrees closely with that expected if a single side-chain were present. The presence of a small percentage (1.7%) of a 2,3-dimethyl ether could arise from under-methylation, but an

alternative explanation is more probable, considering other results. Methylation analyses conducted on purified samples incubated with alpha amylase gave the results shown in Table II.

The treatment with alpha amylase almost completely removed the (1→4) linkages and completely removed the (1→4→6) linkages, showing that these were present in a glucan chain in which most of the linkages had the α configuration.

Periodate oxidation. — Measurements on the purified sample prior to enzymic digestion showed 0.45 mol of periodate consumed and 0.121 mol of formic acid produced, in close agreement with the values predicted on the basis of methylation analysis (0.42 mol of periodate consumed and 0.125 mol of formic acid produced). The periodate consumption in the presence of buffer was 0.47 mol of glucose residues, indicating that there was no over-oxidation. When the periodate-oxidized polymer was reduced and subjected to reoxidation, no further periodate consumption took place. Smith periodate oxidation[23] of the glucan yielded glycerol, a large polymer, and a two-carbon compound identified as glyoxalate, all of which were radioactive. The proportion of the radioactivity present in the three products was 1:40:0.7. There was insufficient mass in the glycerol peak to allow detection with staining reactions, and no erythritol could be detected either by staining, or as radioactivity. Incubation of the purified glucan with alpha amylase caused a decrease in the periodate consumption, to 0.25 mol/glucose residue, which compares with a value of 0.23 mol predicted for a pure (1→3)-glucan having a single side-chain.

Alkaline degradation. — The glucan was rapidly degraded by alkali and only 30% of the original polymer remained after 2 h. Further reaction took place more slowly, with an additional 5% being lost during the next 22 h.

Optical rotation. — The optical rotation of the glucan changed from +2.5 to −7.4° after treatment with alpha amylase.

Enzymic hydrolysis. — Incubation of the purified glucan with alpha amylase showed release of very small amounts of non-radioactive glucose, maltose, and maltriose. In addition, after incubation with alpha amylase, further unlabelled products were detected at the bed volume when the digest was fractionated on a column of Biogel P-30. However, the main mass, and all of the radioactivity, was eluted as a single peak having an elution volume very slightly greater than that of the untreated glucan.

Incubation of the glucan with the (1→3)-β-D-glucan hydrolase of *N. glutinosa* released a series of radioactive products during the 48-h incubation period, as shown in Fig. 1. On the basis of R_{Glc} values (paper chromatography in systems *a* and *b*), the products fell into two distinct groups. Oligosaccharides containing 2–6 glucose residues were linear laminara-oligosaccharides [β-D(1→3) linkages], and the larger oligosaccharides were broken down to smaller ones as the incubation time was increased. However, two larger oligosaccharides containing 7 or more glucose residues broke down very slowly, with very small changes in R_{Glc} value and corresponding increases in the glucose present in the sample. Such products were not present in laminaran controls, and were not members of the linear, laminaran series.

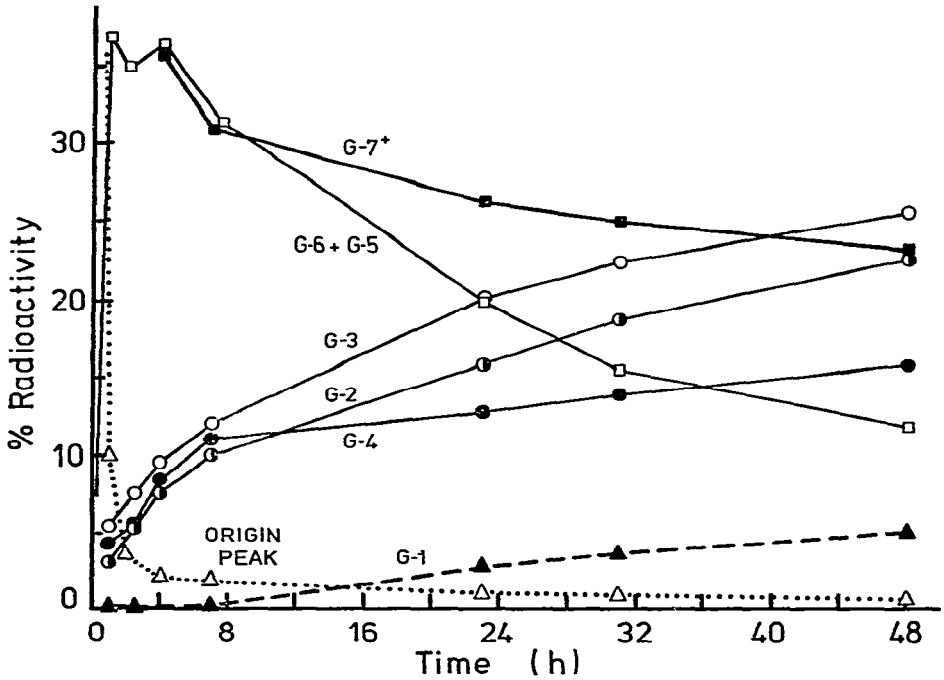

Fig. 1. Kinetics of hydrolysis of *Caulerpa* glucan by *N. glutinosa* endo-(1→3)-β-D-glucan hydrolase. Products were separated by solvent system *a* for 24 h. Components G-1, G-2, G-3, and G-4 were identified as glucose, laminarabiose, laminaratriose, and laminaratetraose, respectively, by using the appropriate standards.

When the products of 48-h incubation of the glucan with the *N. glutinosa* enzyme were separated by gel chromatography, hepta- and hexa-saccharides were the largest fragments that could be identified, although their R_{Glc} values on paper chromatography suggested that they were larger.

Following incubation of the β-D-glucan with this D-glucan hydrolase, 14% of the mass failed to dissolve, but again this insoluble material was not radioactive. When the glucan was incubated with an exo-(1→3)-β-D-glucan hydrolase from *E. gracilis*, the results shown in Fig. 2 were obtained. The number of reducing groups and the amount of glucose present increased almost linearly from 2 to 8 h, by which time all of the original glucan had been degraded. During this period, two other compounds, a trisaccharide and a tetrasaccharide, increased in concentration more slowly. Continuation of the incubation period to 44 h gave no significant alteration to this pattern (Fig. 2).

Gel chromatography of the products of enzymic digestion after 44 h showed that glucose contained 63% of the radioactivity applied, and 54% of the original mass. A shoulder corresponded to laminarabiose and separate peaks to laminaratriose and -tetraose. When separated on paper, each of these fractions contained one other, unidentified, oligosaccharide that was degraded slowly to glucose and gentio-

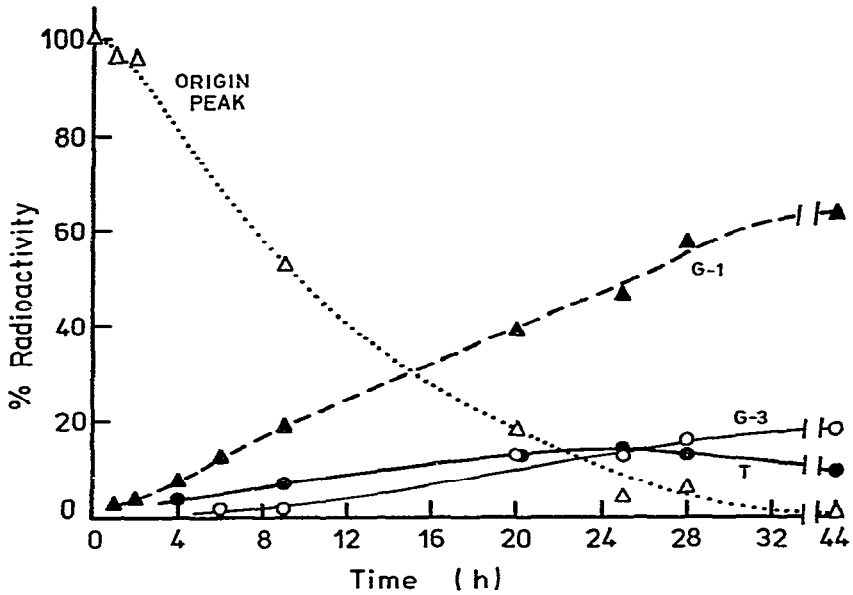

Fig. 2. Kinetics of hydrolysis of *C. simpliciuscula* glucan by an exo-(1→3)-β-D-glucan hydrolase from *Euglena gracilis*. Products were separated by using solvent system *a* for 24 h. The unidentified tetrasaccharide (T) is believed to be a Glc-(1→3)-Glc-(1→3)-Glc-(1→6)-Glc component from its R_{Glc} value.

biose between 24 and 44 h of digestion, suggesting that (1→3) and (1→6) linkages were present. In this separation, some 6% of the mass was eluted as a non-radioactive peak at the void volume.

DISCUSSION

Previous examination of this glucan suggested that it contains a backbone of β-(1→3)-linked D-glucose residues, one or more (1→6)-branch points, and (1→6) or (1→4) linkages within the chain[3].

The methylation analysis and periodate-consumption results from this work show that a single-branch, (1→6) branch-point is present and that the linkages generating the additional periodate consumption were (1→4). These results would be consistent with the polymers being a mixed-linked β-glucan having a single branch point, containing some β-D-(1→4)-linked residues.

However, while the gel-filtration separations, and the ultracentrifugal analysis, suggested that a single compound was present, the results of the enzymic hydrolysis were not consistent with this interpretation. Although digestion with alpha amylase failed to release any radioactive products, it did release unlabelled products. Similarly, the Smith periodate reaction produced no radioactive erythritol, suggesting that no labelled sugars were linked by (1→4) linkages.

The results are consistent with two possible structures: (*A*) a single compound

TABLE III

THE COMPOSITION OF *C. simpliciuscula* GLUCANS CALCULATED FROM THE METHYLATION-ANALYSIS DATA

Compound	*Untreated*		*Digested with alpha amylase*
	(1→4)-Linked compound	*(1→3)-Linked compound*	*(1→3)-Linked compound*
Average percent abundance	23	77	98
No. of non-reducing terminals	3	1.8	2
No. of chain linkages	24	27	24
No. of branch points	2	1.2	1.2
Reducing end-groups	1	1	1
Total d.p.	30	31	28

composed mainly of radioactively labelled β-(1→3)-linked D-glucose residues and a block of unlabelled α-(1→4)-linked glucose residues; (*B*) two separate molecules, a β-(1→3)-linked D-glucan and an α-(1→4)-linked D-glucan. After digestion of the sample with alpha amylase, there is a decrease in periodate consumption, a loss of one of the i.r. bands, (attributed to α linkages), and a decrease in optical rotation. Treatment of the glucan with alpha amylase decreased the content of (1→4) linkages from 19 to 1.2% (Table III). This result shows that the (1→4) linkages can be removed by alpha amylase, and confirms that they were α- rather than β-linked. The molecular weight of the alpha amylase digest was only slightly lower than that of the original glucan, and the decrease, although observed consistently with 3 runs, falls within the experimental error of the measurement. Therefore it seems very probable that the β-glucan fraction, as isolated, contains an α-(1→4)-linked, branched compound in the ratio of three (1→3)-β-D-glucan molecules per (1→4)-α-D-glucan. The composition of the mixture, calculated on the basis of the methylation analysis and by the periodate consumption, is shown in Table III. Had the α-(1→4)-linked D-glucose residues been covalently linked to the (1→3)-β-D-glucan chain, the decrease in apparent molecular weight after digestion by alpha amylase would have been of the order of 23%, and as such would have readily been detected by gel chromatography.

The discrepancy in the specific activity of the glucose residues in the two molecules may readily be understood by examining the kinetics of the labelling of the β-glucan and starch, which has been discussed in earlier reports from our laboratory[1,2]. After 1 h of photosynthesis in $^{14}CO_2$, the β-glucan has almost reached saturation, whereas the ^{14}C content of the starch is still very low. Presumably, the soluble, low-molecular-weight starches are present either as precursors, or more likely, as degradation products of the insoluble starch, and therefore contain no label. Soluble starches have been reported in other species of *Caulerpa*[4,6], but the molecular weight is much higher (15,000 daltons) than the apparent molecular weight of this material, whose molecular weight must be almost identical with that of the β-glucan, namely ~5,000 daltons. This α-glucan appears to associate in a constant (1:3)

proportion with the β-glucan, as we have found almost constant periodate-consumption and methylation-analysis results from 10 separate isolates. It would therefore appear that the association is not random.

The structure of the β-glucan itself, which was the main object of our studies, is therefore considered to be a backbone of β-(1→3)-linked D-glucose residues, with between 1 and 2 β-(1→6)-linked branch points. The alkaline-hydrolysis results and the degradation with *N-glutinosa* enzyme suggests that the maximum length of the side chain is 4 glucose residues. The glucan is freely soluble in water, precipitating only at high concentrations of ethanol, suggesting that the β-(1→3)-chain backbone cannot form a helix, and therefore the branch point(s) are close to the centre of the backbone. This β-D-glucan therefore differs from the β-D-glucans found in other algae, in that it contains no interchain, (1→6) groups, and no mannitol termini. Its structure appears to be closest to that of the mycolaminarans found in *Phytophthora* species[12]. Current work suggests that this β-D-glucan serves as a specific reserve for the synthesis of cell-wall material that is laid down during the final stages of wound healing in this plant.

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